Isolation and culture of inner cell mass cells from human blastocysts

Ariff Bongso[1], Chui-Yee Fong, Soon-Chye Ng and Shan Ratnam

Department of Obstetrics and Gynaecology, National University Hospital, Kent Ridge, Singapore 0511

[1]To whom correspondence should be addressed

Totipotent non-committed inner cell mass (ICM) cells from human blastocysts, if demonstrated to be capable of proliferating *in vitro* without differentiation, will have several beneficial uses, not only in the treatment of neurodegenerative and genetic disorders, but also as a model in studying the events involved in embryogenesis and genomic manipulation. Nine patients admitted to an in-vitro fertilization programme donated 21 spare embryos for this study. All 21 embryos were grown from the 2-pronuclear until blastocyst stages on a human tubal epithelial monolayer in commercial Earle's medium (Medicult, Denmark) supplemented with 10% human serum. The medium was changed after blastocyst formation to Chang's medium supplemented with 1000 units/ml of human leukaemia inhibitory factor (HLIF) and the embryos left undisturbed for 72 h to allow the hatched ICM and trophoblast to attach to the feeder monolayer. Nineteen of the 21 embryos from nine patients produced healthy ICM lumps which could be separated and grown *in vitro*. Two of the lumps differentiated into fibroblasts while the remaining 17 (eight patients) produced cells with typical stem cell-like morphology, were alkaline phosphatase positive and could be maintained for two passages. It was possible to retain the stem cell-like morphology, alkaline phosphatase positiveness and normal karyotype through the two passages in all of them using repeated doses of HLIF every 48 to 72 h. This is the first report on the successful isolation of human ICM cells and their continued culture for at least two passages *in vitro*.
Key words: HLIF/human ES-like cells/ICM cells/tubal epithelial monolayer

Introduction

The human embryo develops from a group of undifferentiated cells into an organism with many specialized cells, tissues and organs. During embryogenesis the fates of cells become gradually restricted as they enter new developmental pathways. In their classical lineage analysis studies in the mouse, Gardner and Papaioannou (1975) described the fate of the inner cell mass (ICM) and trophectoderm. Their main findings were the possibility of a unitary origin of all trophoblast cells, and the formation of the entire fetus and extra-embryonic mesoderm from the non-endoderm cells of the ICM. The ICM of the mammalian blastocyst contains undifferentiated non-committed cells with the potential to enter a full range of developmental pathways and it is well known that as these cells differentiate, they lose the capacity to enter developmental pathways previously open to them (Anderson, 1992). These totipotent embryonic stem (ES) cells, if isolated and made to proliferate without differentiation, could have several uses in not only the treatment of neurodegenerative disorders but also the development of monoclonal antibodies to human proteins for diagnostic and therapeutic use (Edwards, 1992).

The first ES cell lines that were typed and passaged were obtained in the rabbit (Cole *et al.*, 1965a,b). Later, ES cells were isolated from mouse embryos (Evans and Kaufman, 1981; Martin, 1981) and ES-like cells from farm animal embryos (Wilmut *et al.*, 1991). Feeder cells or agents with differentiation inhibitory activity encouraged ES-like cell growth and prevented them from undergoing spontaneous differentiation (Nichols *et al.*, 1990). Cells of the STO line, which are thioguanine- and ouabain-resistant, have been successfully used as feeders for differentiation of clonal lines of murine teratocarcinoma cells. Cell division of these feeder cells was prevented with mitomycin C (Martin and Evans, 1975). Medium conditioned by Buffalo rat liver (BRL) cells prevented the spontaneous differentiation of embryonal carcinoma and ES cells (Handyside *et al.*, 1987; Smith and Hooper, 1987), and myeloid leukaemia inhibitory factor (LIF) (Williams *et al.*, 1988) was also used to maintain the stem cell phenotype of ES cells in the mouse. In the murine species, ICM cells are able to colonize the somatic tissues of chimaeric mice following injection of such cells into blastocysts and return of the micromanipulated embryos to pseudopregnant recipients (Bradley *et al.*, 1984; Robertson and Bradley, 1986; Beddington and Robertson, 1989). However, Notarianni *et al.* (1990) pointed out that the methods devised for ES cell production for one species may not be directly applicable to another species, the reasons for this being (i) the differences in the timing of differentiation of blastocysts between species and (ii) the development of ICM being significantly different between murine and ungulate species. However, it has been shown that in the human, unlike the mouse, 42% of normally fertilized embryos reached the blastocyst stage on day 5 or 6 post-insemination and the proportion of ICM cells remained higher despite cell death in both ICM and trophectoderm (Hardy *et al.*, 1989). In mouse embryos, proliferation of the ICM occurs rapidly, whereas in ungulates the ICM forms a mitotically quiescent embryonic disk (Piedrahita *et al.*, 1990). Growth characteristics and morphology of ES-like cells may thus differ between species. We report here the use

of a human oviductal epithelial feeder layer together with human leukaemia inhibitory factor (HLIF) to isolate and grow ES-like cells from human embryos in culture for at least for two passages.

Materials and methods

Patients and embryos

For this study, nine patients aged 35−41 years enrolled in an assisted reproduction programme donated 21 spare embryos. They underwent ovarian stimulation using one of two hormonal regimes: (i) follicle stimulating hormone (FSH) + human menopausal gonadotrophin (HMG) or (ii) gonadotrophin-releasing hormone + HMG. Consent to carry out this study was approved by the hospital ethical committee based on the guidelines on Assisted Reproductive Technology of the Ministry of Health, Singapore that experimentation of human embryos up to day 14 of embryonic growth may be allowed.

Feeder layers

Embryos were grown and allowed to hatch on passaged human Fallopian tubal epithelial monolayers which acted as feeder layers. Ampullary epithelial cells separated from healthy Fallopian tubes of cycling pre-menopausal women undergoing hysterectomy were used to establish primary cultures and cell lines according to the method of Bongso et al. (1989a). The patients donating Fallopian tubes consented to human immunodeficiency virus (HIV) antibody tests at the time of hysterectomy and 6 months later. Cell cultures were quarantined during this 6 month period by freezing in liquid nitrogen using a slow freezing method with 10% dimethyl sulphoxide (DMSO) as cryoprotectant. Once both tests were negative, the cells were thawed and monolayers established. Monolayers were also screened for hepatitis B and other microbes using conventional microbiological tests. For culture of embryos, cells with epithelioid cell morphology that were cytokeratin positive were used as feeder layers for this study.

At 24 h prior to oocyte retrieval, the monolayers were detached from their plastic flasks with 0.5% trypsin + 0.53 mM EDTA [Grand Island Biological Company (Gibco), Grand Island, NY, USA] at 37°C in a 5% CO_2, 95% air atmosphere for 5−10 min. The detached cells were transferred to sterile plastic tubes (Falcon, MD, USA) and centrifuged at 300 g for 5 min. The cell pellet was washed at least twice with Hank's balanced salt solution (Gibco, New York, USA) and finally resuspended in IVF culture medium (Medicult, Copenhagen, Denmark). Medicult is a commercial medium containing Earle's balanced salt solution supplemented with human serum albumin, insulin and antibiotics. Cell resuspension was carried out many times to allow the cells to break up into single cells or clusters. Then ~ 100 000 cells were seeded into 0.8 ml of IVF culture medium (Medicult) + 10% heat-inactivated human serum in each well of 4-well plastic dishes (Nunclon, Copenhagen, Denmark) and the dishes incubated at 37°C in a 5% CO_2, 95% air atmosphere. The human serum was prepared from blood collected from fasted patients undergoing ovarian stimulation just after they had been administered human chorionic gonadotrophin for triggering ovulation and just prior to oocyte retrieval. Confluent monolayers were ready 48 h later for the culture of pronuclear-stage human embryos.

Embryo culture

The 21 spare embryos at the 2-pronuclear stage were placed on the ampullary cell feeder layers. The dishes were incubated at 37°C in a 5% CO_2, 95% air atmosphere and the medium replaced every 48 h with fresh Medicult medium. Once cavitating blastocysts were formed, the medium was changed to Chang's medium (Irvine Scientific, USA) supplemented with 10% human serum and 1000 units/ml HLIF (Sigma, MO, USA). Chang's medium is an enriched complex medium containing amino acids, polypeptides, vitamins, ribo- and deoxyribonucleosides, hormones and trace elements, while HLIF has been proved to prevent cell differentiation. The blastocysts were left undisturbed over the next 24−48 h to allow hatching and for the ICM lumps to attach to the monolayer (Figure 1). The Chang's medium with HLIF was replaced every 48 h thereafter.

Isolation and culture of ES-like cells

The ICM lumps attached to the monolayer and large trophoblast-like cells began to spread out from the peripheral regions of each lump on day 8 or 9 post-insemination (Figure 2). The ICM cells piled up vertically as lumps and underwent degeneration in 4−5 days if they were not separated from the trophoblast and feeder layer, and they were then re-cultured (Figure 3). Separation of the ICM lump was carried out using the blunt heat-sealed end of a finely drawn-out Pasteur pipette. Using a stereomicroscope at high magnification, separation of only the upper parts of the ICM lump was done very carefully, without removing tubal cells from the feeder layer. It was easy to recognize any tubal cell contamination from their typical epithelioid cell morphology, and the growth of tubal cells was so prolific that they would outgrow any ES-like cells. The lumps were picked up with a minimal volume of medium and transferred to a 50 μl drop of 0.5% trypsin + 0.53 mM EDTA under mineral oil (Sigma) and incubated for 10 min for partial cell disaggregation at 37°C in a 5% CO_2, 95% air atmosphere. The further action of trypsin was prevented

Fig. 1. Human blastocysts (144 h post-insemination) hatching on human tubal ampullary epithelial monolayer (×100).

A.Bongso *et al.*

by resuspending the cells in another 50 μl of Chang's medium + 10% human serum + 1000 units/ml HLIF without cell monolayers. Once several colonies from each blastocyst had formed on the plastic in 10–14 days, the cells were subcultured.

Only colonies having the typical stem cell-like morphology were subcultured. The subculturing method was similar to the disaggregation of ICM cells using trypsin + EDTA. Cell colonies continued to form quite well on plastic in the presence of HLIF in Chang's medium supplemented with 10% human serum. The HLIF was replaced every 48 h to prevent cell differentiaton. Once the wells became fairly confluent (8–10 days), the cells were subcultured again and when ~2–3×10⁶ cells per blastocyst were available, they were frozen using the conventional slow freezing machine method with 10% DMSO as cryoprotectant.

Identification of ES-like cells

Confirmation that the ICM cells had not differentiated and were ES-like cells was based on cell morphology analysis using phase-contrast and Nomarski's optics, as reported for other species, and the alkaline phosphatase test, which is a biochemical marker for stem cells. Histochemical staining for alkaline phosphatase was carried out as described by Donovan *et al.* (1986). Cells showing typical stem cell-like morphology from primary and subcultures were first fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were then washed with distilled water and the fixed cultures incubated for 15 min at 37°C in distilled water containing 1 mg/ml Fast Red TR salt and 40 μl/ml napthol AS-MX phosphate (Sigma) at a pH of 8.4. Washing the cells with PBS stopped the reaction after 15 min.
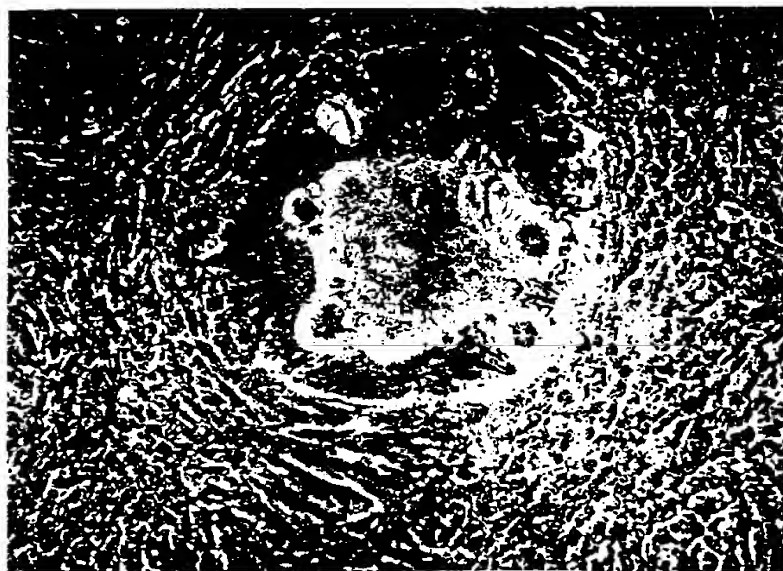

Fig. 2. Inner cell mass (ICM) lump with peripheral trophoblast-like cell outgrowths 2 days after hatching. Note healthy ICM lump (white translucent centre) (×200).
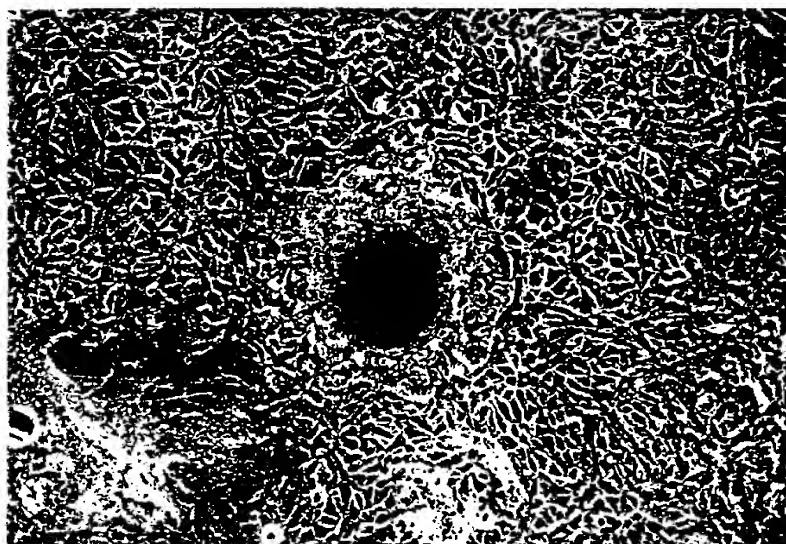

Fig. 3. Inner cell mass lump with peripheral trophoblast-like cells attached to ampullary epithelial monolayer 5 days after hatching. Note degeneration in centre of lump (black centre) as a result of delay in separation from monolayer and re-culturing (×200).
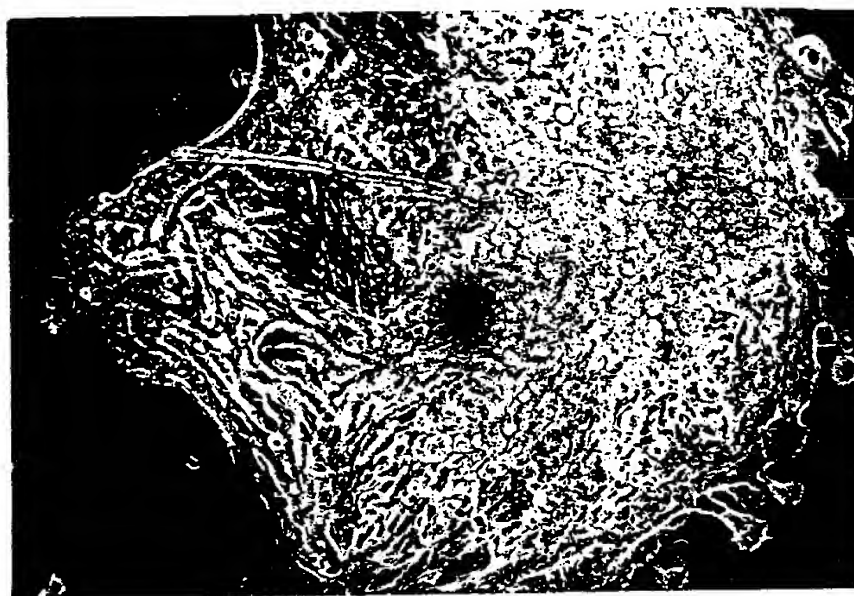
Fig. 4. Separated and disaggregated inner cell mass lump growing on plastic in the presence of HLIF + Chang's medium + human serum, 10 days post-insemination. Note translucent ES-like cells forming circular nests (×200).

Fig. 5. Periphery of inner cell mass lump spreading in primary culture. Note small white ES-like cells with large nucleus and scant cytoplasm (×400).

Red staining of cells indicated a positive alkaline phosphatase reaction. The red precipitate was not stable over long-term storage.

To check whether the genotypes of the cells were normal and had not changed, the conventional method for chromosome analysis on human amniotic fluid cells was applied to the cell cultures. Aliquots of actively mitotic cells with classical ES cell-like morphology were separated from each ICM culture of each embryo and exposed to 10 μg/ml colcemid solution (Gibco) for 2 h at 37°C in a 5% CO_2, 95% air atmosphere to arrest all dividing ES-like cells at metaphase. The cells were then treated with a hypotonic solution of 0.075 M potassium chloride to rupture the cells. Chromosomes and nuclei were then fixed with glacial acetic acid:methanol (1:3) for 30 min at 4°C. Drops of cells from the resuspended cell suspension were placed on pre-cleaned grease-free glass slides, air-dried and then subjected to the trypsin-Giemsa banding method to identify minor chromosome rearrangements. Good-quality, complete-banded metaphases were photographed and karyotypes constructed and analysed by computer automated chromosome analysis (Cytovision, Applied Imaging, UK).

Epithelioid morphology was evaluated using the standard immunofluorescent staining method specific for cytokeratin. Cell cultures were rinsed with PBS and fixed in 4% formaldehyde
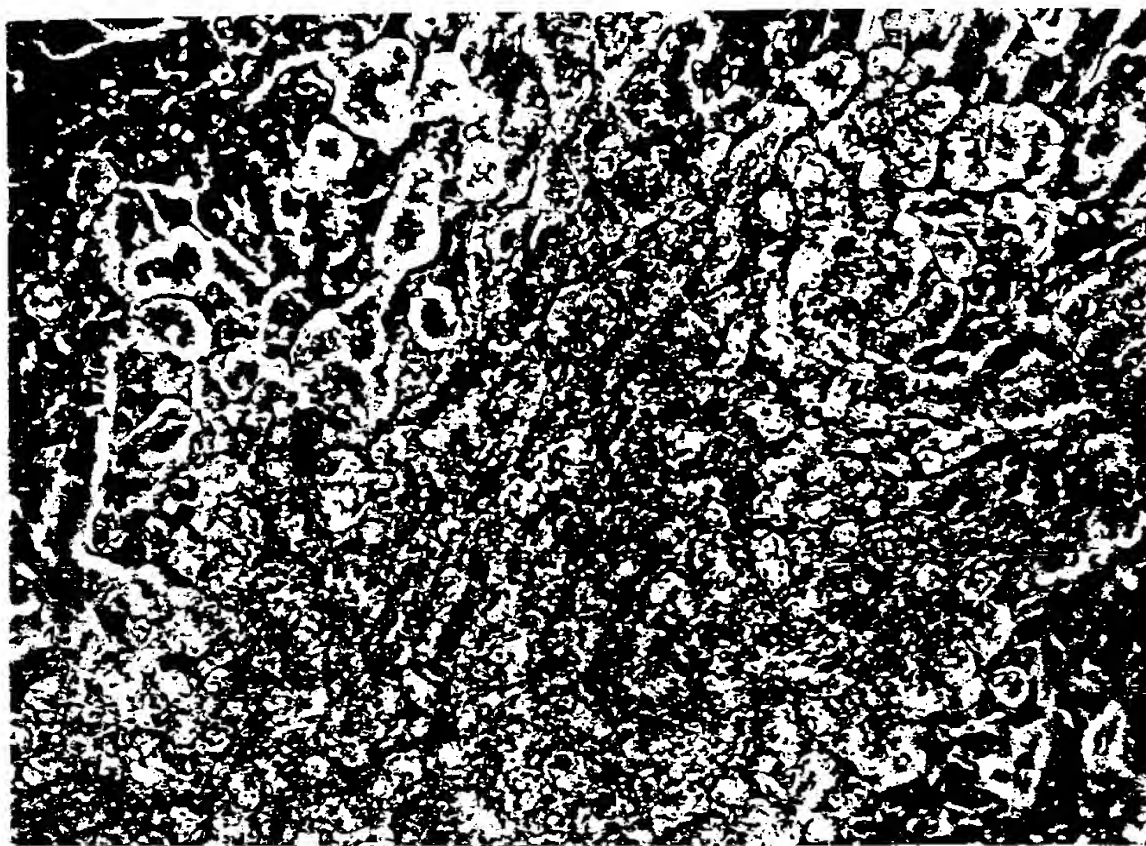
2113

A.Bongso et al.

Fig. 6. High magnification of inner cell mass cells. Note epithelioid monolayer with circular small and large cell nests typical of ES-like cells (×600).

in PBS for 30 min at room temperature. The cells were rinsed with PBS and permeabilized for 20 min with 0.5% (v/v) Triton X-100 in PBS. The cell cultures were then incubated cell side down in a monoclonal anti-cytokeratin (Sigma) diluted 1:20 (v/v) in PBS for 90 min at room temperature in a humidified chamber. The cells were then washed with PBS and incubated for 60 min in FITC-conjugated anti-IgG diluted 1:50 (v/v) in PBS. The cultures were washed in PBS and mounted in glycerol-PBS for viewing under a Zeiss fluorescent microscope equipped with epifluorescence and FITC excitation.

Results

Nineteen embryos (from nine different patients) out of the 21 spare embryos donated produced healthy ICM lumps which could be successfully separated and maintained in cell culture through at least two subcultures without differentiation. The ICM cells in two of the 19 embryos (one patient) became differentiated into spindle-shaped fibroblasts, while those of the remaining 17 embryos (eight patients) produced the typical stem cell-like morphology reported for other species. A typical ICM cell colony and monolayer are shown in Figure 4. The cells had an epithelioid morphology with large nuclear to cytoplasmic ratios. Each cell was small, with a large nucleus and minimal cytoplasm (Figure 5), and the nuclei contained one or more prominent nucleoli. The cells were actively mitotic and tightly packed in small nests or colonies in which it was difficult to recognize individual cells.

Figure 6 illustrates the progressive growth of a colony in the absence of cell differentiation, and this represents a true colony as observed in other species.

The ICM cells were stable to subculturing in the undifferentiated state in the presence of regular doses of HLIF. and they maintained morphology similar to that of ICM cells in primary culture in all 17 embryos of the eight patients. After the second subculture, the cells differentiated into fibroblasts or died. All ICM cells showing typical stem cell-like morphology in primary and first and second subcultures were alkaline phosphatase positive. Giemsa-banded chromosome analysis on 15−20 metaphases per primary and passaged ICM culture for each embryo showed normal 46XX karyotypes for eight, and normal 46XY karyotypes for nine of the 17 embryos (Figures 7 and 8).

Discussion

To the best of our knowledge, this is the first report on the possibility of developing non-committed ICM cells from late preimplantation human embryos, although cell lines have been isolated from embryos of the mouse (Evans and Kaufman, 1981: Martin. 1981). pig (Piedrahita et al., 1988: Evans et al., 1990: Notarianni et al., 1990), golden hamster (Doetschmann et al., 1988), sheep (Handyside et al., 1987; Notarianni et al., 1990: Piedrahita et al., 1990), cow (Evans et al., 1990), rabbit (Giles et al., 1993) and mink (Sukoyan et al., 1993).
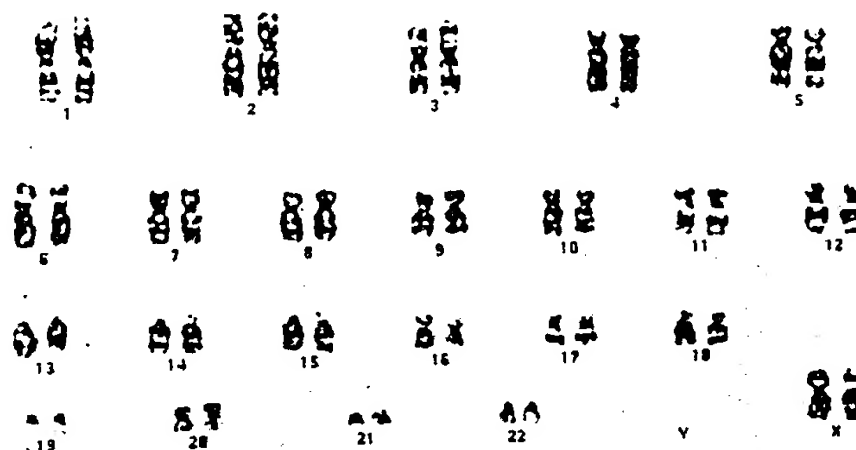
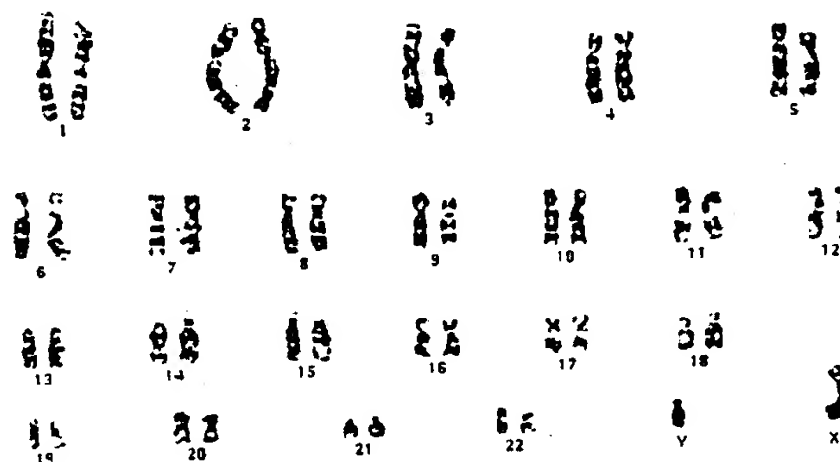Fig. 7. Giemsa-banded karyotype of an ES-like cell from primary culture showing a normal 46XX karyotype.

Fig. 8. Giemsa-banded karyotype of an ES-like cell from the first passage showing a normal 46XY karyotype.

The cell colonies in this study showed a remarkable resemblance to those formed from embryos of other species. The cell lines isolated from agricultural animals were of two types; some were similar in appearance to mouse ES cells while others were epithelial-like. The human ES-like cells in this study were clear-cut epithelioid-like cells growing in typical colonies or nests resembling very closely the morphology of the murine ES cells and human embryonal carcinoma cells observed by Pera *et al.* (1989). Also, proliferation of the ICM occurred very rapidly in the human cells, which is the same as is observed in mouse but not ungulate cells.

It has been suggested that the poor success rates in developing ES cells in species other than mice appeared to be due to either fundamental biological differences between species or simply to technical factors associated with the optimization of culture conditions (Piedrahita *et al.*, 1990). In the human, it appears that human tubal ampullary feeder cells together with HLIF in an enriched medium such as Chang's supplemented with human serum was an ideal in-vitro system to initiate the primary growth of ICM cells. Furthermore, a feeder layer was not necessary for subsequent subcultures if HLIF was present in the medium. Our preliminary studies prior to this report demonstrated clearly that,

2115

A.Bongso *et al.*

in the absence of an initial feeder layer and subsequent HLIF, the ICM cells were difficult to sustain or always differentiated into fibroblast-like cells. It has been shown recently that mouse ES cells can be isolated by placing ICM cells directly into murine leukaemia inhibitory factor (mLIF) supplemented medium in the absence of feeder cells (Nichols *et al.*, 1990; Pease *et al.*, 1990). Experiments in which mLIF or HLIF was used to assist isolation of ES cells from domestic livestock species have been unsuccessful (Anderson, 1992). It therefore appears that the action of murine or human LIF on ICM cells may be species dependent.

Human epithelioid monolayers were used in this study instead of the commonly used STO mouse fibroblasts and other cell types because our previous work and that of others has shown that the presence of such helper cells in the in-vitro system can be very effective in generating viable blastocysts (Bongso *et al.*, 1989b; Yeung *et al.*, 1992). Since STO fibroblasts were not used in this study it is not possible to conclude whether or not they would be equally effective as feeder layers. A feeder cell type similar to the species of the embryo may be more ideal than that of a heterologous species. Interestingly, Anderson *et al.* (1992) showed that hatched bovine blastocysts attached to bovine oviductal epithelial cell monolayers in Dulbecco's modified Eagle's medium but failed to proliferate. Perhaps the use of an enriched medium like Chang's medium supplemented with serum may have helped cell proliferation.

Totipotent cells do reside in the ICM of animal embryos. ICM transplantation has resulted in the birth of chimaeric offspring in mice (Bradley *et al.*, 1984; Robertson and Bradley, 1986; Beddington and Robertson, 1989; Lallemand and Brulet, 1990), pigs (Anderson, 1992) and sheep (Butler *et al.*, 1987). Bovine chimaeras have been produced by blastocyst injection using ICM cells (Summers *et al.*, 1985). Recently, the developmental ability of enucleated mouse oocytes that had received murine ES cells was examined. Electrofusion was necessary for the oocytes to form nuclei and cleave to blastocyst stages. Although implantation sites were observed after transfer of embryos to pseudopregnant females, no live fetuses were obtained (Tsunoda and Kato, 1993).

Criteria for proper identification of ES-like cell colonies are usually based on the morphology exhibited by murine ES cells, and using human ES-like cells to produce germline chimaeras is not possible for ethical reasons. However, other useful applications for human ES-like cells can be envisaged, particularly if differentiation can be directed along chosen pathways. Since the primitive streak starts forming around day 14 of gestation it has been argued that life begins only then, and as such the ethical guidelines in Singapore allow experimentation and manipulation of human embryos up to day 14. The culture of embryonic cells after the 14th day should not face ethical problems since the organization of the embryo is lost in the process of cell culture, and the ICM or ES-like cells are just a monolayer of cells with no potential of becoming a human. However, theoretically, it is possible that such cells (because of their totipotency) could be activated to produce clones after insertion into enucleated oocytes. Utmost caution should thus be taken to minimize the risks of their abuse, and the uses of such cells must be restricted to somatic cell therapy and as models for embryonic differentiation. This issue needs careful deliberation and clarification.

The overall objective of the experiments in the present study was to measure survival in culture of human ICM cells from blastocysts produced by procedures for IVF. We conclude that totipotent embryonic lineages can be derived from the human and can be maintained in culture at least for a few subcultures without differentiation. More work needs to be done to encourage further growth so as to establish a cell line of ES cells. Reasonable cell numbers could thus be generated for intra-uterine transfer into fetuses suffering from incurable genetic disorders. It would be interesting to also investigate the range of differentiated cell types that could be formed from such pluripotent ES-like cells. Work is in progress to examine these cells as embryoid bodies after transfer to ectopic sites in nude mice.

Acknowledgements

References

Anderson,G.B. (1992) Isolation and use of embryonic stem cells from livestock species. *Anim. Biotechnol.*, **3**, 165–175.

Anderson,G.B., Behboodi,E. and Pacheco,T.V. (1992) Culture of inner cell masses from in vitro-derived bovine blastocysts. *Theriogenology*, **37**, 187.

Beddington,R.S.P. and Robertson,E.J. (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development*, **105**, 733–737.

Bongso,A., Ng,S.C., Sathananthan,H., Ng,P.L., Rauff,M. and Ratnam,S.S. (1989a) Establishment of human ampullary cell cultures. *Hum. Reprod.*, **4**, 486–494.

Bongso,A., Ng,S.C., Sathananthan,H., Ng,P.L., Rauff,M. and Ratnam,S.S. (1989b) Improved quality of human embryos when cocultured with human ampullary cells. *Hum. Reprod.*, **4**, 706–713.

Bradley,A., Evans,M., Kaufman,M.H. and Robertson,E. (1984) Formation of germ-line chimaeras from embryo derived teratocarcinoma cell lines. *Nature*, **309**, 255–256.

Butler,J.E., Anderson,G.B., BonDurant,R.H., Pashen,R.L. and Penedo,M.C.T. (1987) Production of ovine chimaeras by inner cell mass transplantation. *J. Anim. Sci.*, **65**, 317–324.

Cole,R.J., Edwards,R.G. and Paul,J. (1965a) Cytodifferentiation in cell colonies and cell strains derived from cleaving ova and blastocysts of the rabbit. *Exp. Cell Res.*, **37**, 501–504.

Cole,R.J., Edwards,R.G. and Paul,J. (1965b) Cytodifferentiation and embryogenesis in cell colonies and tissue cultures derived from ova and blastocysts of the rabbit. *Dev. Biol.*, **13**, 385–407.

Doetschman,T., Williams,P. and Maeda,N. (1988) Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev. Biol.*, **127**, 224–227.

Donovan,P.J., Stott,D., Cairns,L.A., Heasman,J. and Wylie,C.C. (1986) Migratory and postmigratory mouse primordial germ cells behave differently in culture. *Cell*, **44**, 831–838.

Edwards,R.G. (1992) Differentiation and transplantation of embryonic cells in mammals. In Edwards,R.G. (ed.), *Fetal Tissue Transplants in Medicine*. Cambridge University Press, Cambridge, pp. 1–50.

Evans,M.J. and Kaufman,M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature*, **292**, 154–156.

Evans,M.J., Notarianni,E., Laurie,S. and Moor,R.M. (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. *Theriogenology*, **33**, 125–128.

Gardner,R.L. and Papaioannou,V.E. (1975) Differentiation in the

trophoectoderm and inner cell mass. In Balls,M. and Wild,A.E. (ed.), *Early Development of Mammals*. Cambridge University Press, Cambridge, pp. 107–132.

Giles,J.R., Yang,X., Mark,W. and Foote,R.H. (1993) Pluripotency of cultured rabbit inner cell mass cells detected by izozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae. *Mol. Reprod. Dev.*, **36**, 130–138.

Handyside,A.H., Hooper,M.L., Kaufman,M.H. and Wilmut,I. (1987) Towards the isolation of embryonal stem cell lines from the sheep. *Roux's Arch. Dev. Biol.*, **196**, 185–190.

Handyside,A.H., O'Neill,G.T., Jones,M. and Hooper,M.L. (1989) Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line. *Roux's Arch. Dev. Biol.*, **198**, 48–55.

Hardy,K., Handyside,A.H. and Winston,R.M.L. (1989) The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. *Development*, **107**, 597–604.

Lallemand,Y. and Brulet,P. (1990) An in situ assessment of the routes and extent of colonization of mouse embryos by embryonic stem cells and their descendents. *Development*, **110**, 1241–1248.

Martin,G.R. (1981) Isolation of a pluripotential cell line from early mouse embryos cultured in medium conditioned with teratocarcinoma cells. *Proc. Natl. Acad. Sci. USA*, **78**, 7634–7639.

Martin,G.R. and Evans,M.J. (1975) Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. USA*, **72**, 1441–1445.

Nichols,J., Evans,E.P. and Smith,A.G. (1990) Establishment of germline-competent embryonic stem (ES) cells using differentiation-inhibiting activity/LIF. *Development*, **110**, 1341–1348.

Notarianni,E., Laurie,S., Moor,R.M. and Evans,M.J. (1990) Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *J. Reprod. Fertil.*, Suppl., **41**, 51–56.

Notarianni,E., Galli,C., Laurie,S., Moor,R.M. and Evans,M.J. (1991) Derivation of pluripotent embryonic cell lines from the pig and sheep. *J. Reprod. Fertil.*, Suppl., **43**, 225–260.

Pease,S., Braghetta,P., Gearing,D., Grail,D. and Williams,R.L. (1990) Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukaemia inhibiting factor (LIF). *Dev. Biol.*, **141**, 344–352.

Pera,M.F., Cooper,S., Mills,J. and Parrington,J.M. (1989) Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. *Differentiation*, **42**, 10–23.

Piedrahita,J.A., Anderson,G.B., Martin,G.R., BonDurant,R.H. and Pashen,R.L. (1988) Isolation of embryonic stem cell-like colonies from porcine embryos. *Theriogenology*, **29**, 286.

Piedrahita,J.A., Anderson,G.B. and BonDurant,R.H. (1990) On the isolation of embryonic stem cells: comparative behaviour of murine, porcine and ovine embryos. *Theriogenology*, **34**, 879–901.

Robertson,E.J. and Bradley,A. (1986) Production of permanent cell lines from early embryos and their use in studying developmental problems. In Rossant,J. and Pedersen,R.A. (eds), *Experimental Approaches to Mammalian Embryonic Development*. Cambridge University Press, New York, pp. 475–508.

Smith,A.G. and Hooper,M.L. (1987) Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev. Biol.*, **121**, 1–9.

Sukoyan,M., Vatolin,S.Y., Golubitsa,A.N., Zhelezova,A.I., Semenova,L.A. and Serov,O.L. (1993) Embryonic stem cells derived from morulae, inner cell mass and blastocysts of mink: comparisons of their pluripotencies. *Mol. Reprod. Dev.*, **36**, 148–158.

Summers,P.M., Shelton,J.M. and Bell,K. (1985) Synthesis of primary Bos taurus–Bos indicus chimaeric calves. *Anim. Reprod. Sci.*, **6**, 91–102.

Tsunoda,Y. and Kato,Y. (1993) Nuclear transplantation of embryonic stem cells in mice. *J. Reprod. Fertil.*, **98**, 537–540.

Williams,R.L., Hilton,D.J., Pease,S., Wilson,T.A., Stewart,C.L., Gearing,D.P., Wagner,E.F., Metcalf,D., Nicola,N.N. and Gough,N.M. (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*, **336**, 684–692.

Wilmut,I., Hooper,M.L. and Simons,J.P. (1991) Genetic manipulation of mammals and its application in reproductive biology. *J. Reprod. Fertil.*, **92**, 245–279.

Yeung,W.S.B., Ho,P.C., Lau,E.Y.L. and Chan,S.T.H. (1992) Improved development of human embryos in vitro by a human oviductal cell coculture system. *Hum. Reprod.*, **7**, 1144–1149.